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Characterization of Low Virulent Strains of Highly Pathogenic A/Hong Kong/156/97 (H5N1) Virus in Mice after Passage in Embryonated Hens' Eggs

Yasuaki Hiromoto, Takehiko Saito, Stephen Lindstrom, and Kuniaki Nerome[1]

Department of Virology I, National Institute of Infectious Diseases, 23-1, Toyama 1-chome, Shinjuku-ku, Tokyo 162-8640, Japan

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Avian influenza A H5N1 viruses were isolated from humans for the first time in Hong Kong in 1997. The virulence of A/Hong Kong/156/97 (HK156) strain in mice was found to change significantly depending on the passage history of the virus. Madin-Darby canine kidney (MDCK) cell-grown parental virus and three of its clones derived from mouse brain showed high pathogenicity in mice after intranasal or intracerebral infection. In contrast, the egg-derived parental virus HK156-E3 and its cloned viruses were markedly less pathogenic in mice. It appeared that differences in pathogenicity among viruses derived from MDCK cells and eggs were due to their ability or inability to disseminate from the lungs to the brain. Sequence analysis of the entire protein coding regions of all eight RNA genome segments revealed a total of six conserved amino acid differences in the HA1 domain (residue 211) of the HA protein, as well as the PB1 (residues 456 and 712), PA (residue 631), NP (residue 127), and NS1 (residue 101) proteins that correlated with observed changes in virulence and neurovirulence of HK156 virus in mice. Thus it was evident that the passaging of HK156 in embryonated eggs led to the adaptation and selection of variants demonstrating markedly decreased pathogenicity and neurovirulence in mice that appeared to be attributable to specific amino acid changes in the HA and internal proteins.

INTRODUCTION

Influenza A H5N1 virus appeared suddenly in humans in Hong Kong in 1997, causing a sensation among public health authorities around the world (de Jong *et al.,* 1997). Despite inefficient human-to-human transmission, this virus was highly pathogenic (33% mortality rate), with all deaths diagnosed in patients with multiorgan failure, suggesting systemic spread of the virus (MMWR, 1997, 1998; Yuen *et al.,* 1998). For instance, A/Hong Kong/156/97 (HK156) influenza A virus caused a fatal infection in a 3-year-old boy who developed Reyes' syndrome and multiorgan failure (Yuen *et al.,* 1998). Phylogenetic analysis of human isolates suggested that all gene segments originated from an avian virus (Claas *et al.,* 1998; Suarez *et al.,* 1998). These viruses were also characterized by multiple basic amino acids adjacent to the cleavage site of the hemagglutinin (HA) (Claas *et al.,* 1998; Subbarao *et al.,* 1998), which have been reported to be critical determinants for virulence in chickens (Kawaoka and Webster, 1988). Although it is still uncertain as to whether the existence of multiple basic amino acids plays a role in virulence in humans and nonhuman mammals, Hong Kong H5N1 viruses, including HK156, have been reported to cause fatal infection in mice (Gao *et al.,* 1999; Gubareva *et al.,* 1998; Lu *et al.,* 1999; Shortridge *et al.,* 1998).

Because human influenza viruses are generally nonpathogenic in mice, mouse-adapted human influenza A virus has been used to analyze mechanisms of virulence or neurovirulence under experimental conditions. From a series of analyses, it has been concluded that virulence factors in mice involve not only the HA but also other genes (reviewed by Ward, 1997). It was therefore significant that HK156 virus was shown to cause lethal infection in mice without prior adaptation in mice (Gao *et al.,* 1999; Lu *et al.,* 1999; Shortridge *et al.,* 1998).

HK156 virus was originally isolated in Madin-Darby canine kidney (MDCK), and subsequent passages have been performed in the above cultured cells (Subbarao *et al.,* 1998). It has been shown that influenza viruses may be affected by the culture system (Gubareva *et al.,* 1998; Ito *et al.,* 1997b; Katz *et al.,* 1987; Meyer *et al.,* 1993). For instance, after growth adaptation of influenza virus in different host systems, specific amino acid changes in the HA molecule have been observed in the HA molecule, mainly around the receptor binding site (Gubareva *et al.,* 1998; Ito *et al.,* 1997b; Katz *et al.,* 1987; Meyer *et al.,* 1993). Even though it is well known that the HA plays an important role in host specificity of influenza virus (Naeve *et al.,* 1984; Vines *et al.,* 1998), it has been reported that HK156 and chicken H5N1 viruses isolated in Hong Kong possessed an HA with receptor specificity typical of avian viruses (Matrosovich *et al.,* 1999).

In this study, we used a mouse model to compare the growth characteristics as well as the virulence of two variants of HK156 with different passage histories that

[1] To whom reprint requests should be addressed. Fax: 81-3-5285-1155. E-mail: knerome@nih.go.jp.

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were propagated in MDCK cells or embryonated hens' eggs, respectively. In the present study, we describe the attenuation of HK156 virus through passaging in eggs and investigate possible mechanisms of attenuation using cloned viruses demonstrating high and low virulence in mice.

RESULTS

Attenuation of MDCK-derived HK156 virus in mice through passage in embryonated eggs

To compare the relative virulence of viruses with different passage histories, groups of mice were inoculated intranasally (i.n.) with 2×10^5 plaque-forming units (pfu) of each virus passaged in MDCK cells or embryonated eggs and monitored for 14 days. As shown in Fig. 1a, MDCK-derived parental virus (HK156-CK) caused lethal infection in mice that demonstrated 0% survival by day 7 postinfection (p.i.). This was in contrast to mice infected with egg-passaged virus (HK156-E3) that showed 80% survival at day 8 p.i. and 40% survival after day 12 p.i. Observed differences in pathogenicity between these two viruses was supported by contrasting survival rates among mice after intracerebral (i.c.) infection (Fig. 1b). In fact, mice infected i.c. with HK156-CK showed 0% survival at day 8 p.i., whereas whose infected with HK156-E3 demonstrated 100% survival after 14 days p.i.

Differences in tissue tropism between HK156-CK and HK156-E3

Differences among survival rates after i.n. or i.c. infection by HK156-CK and HK156-E3 led us to investigate the growth characteristics of these viruses in the lungs, brain, liver, and kidney of mice (Table 1). After i.n. inoculation, virus was detected on day 2 p.i. in the lungs of one mouse infected with HK156-CK. On day 4 p.i., both viruses were found to replicate in the lungs, although HK156-E3 titers were lower than those of HK156-CK. Although virus was isolated at low titers from the liver and kidney of one mouse infected with HK156-E3 strain at day 4 p.i., virus could no longer be detected from these organs at day 6 p.i. However, differences in tissue tropism of both viruses became particularly apparent at day 6 p.i., when approximately 10^3 pfu/ml virus was isolated from the brain of two mice infected with HK156-CK. In contrast, virus could not be detected from the brain of mice infected with HK156-E3.

To investigate the ability of both viruses to replicate in mouse brain, mice were infected i.c. with 1×10^4 pfu of virus and examined on day 4 p.i. (Table 2). Interestingly, both viruses were found to replicate in mouse brain, although virus titers of HK156-E3 infected mice were approximately 2 log units lower than those infected with HK156-CK. To confirm that isolation of virus from brain was not a reflection of surviving residual virus inoculate, mice were also infected i.c. with a non-neurovirulent human H3N2 virus (Aichi68). Subsequently, Aichi68 was not detected in the brain of mice, indicating that virus titers of the above pathogenic and low pathogenic viruses reflected the replication of these viruses in mouse brain.

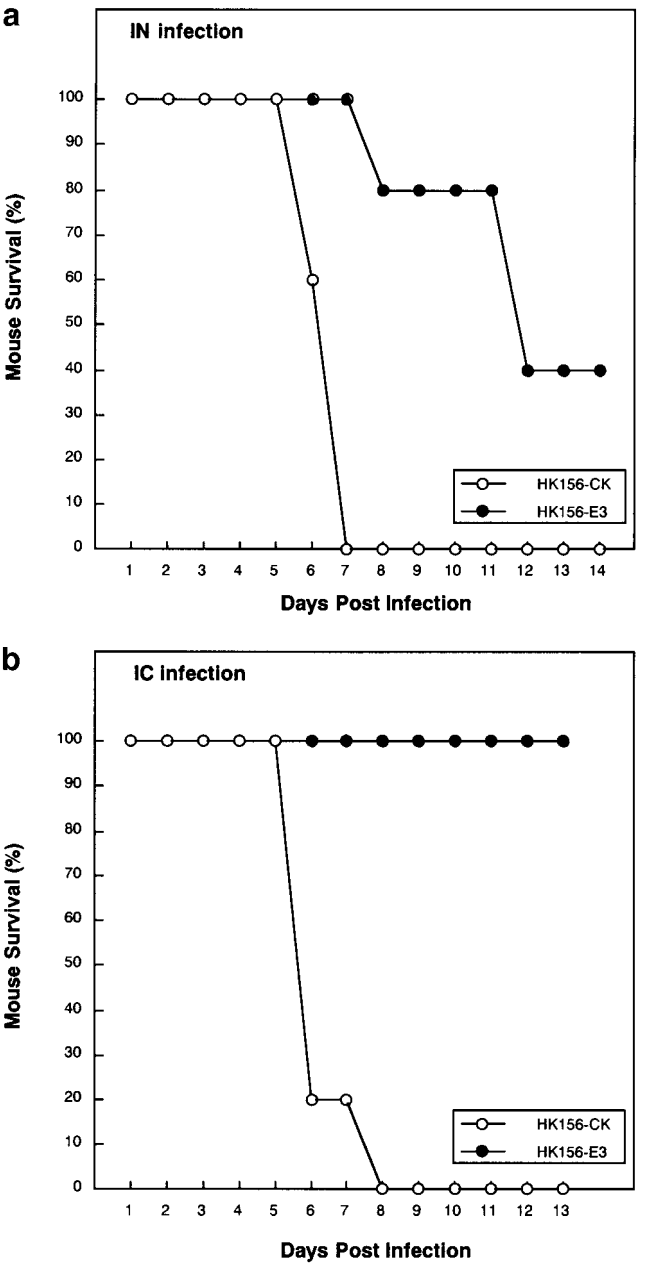

FIG. 1. Survival rates of ddY mice infected with HK156-CK or HK156-E3. Groups of five mice were infected i.n. with 2×10^5 pfu (a) and i.c. with 1×10^4 pfu (b).

Growth behavior of viruses in tissue culture and eggs

To understand observed variability among high and low virulent populations of HK156, viruses isolated on

TABLE 1

Comparative Analysis of HK156 in Mouse Organs after i.n. Infection[a]

Days p.i.	Virus	Virus infectivity (log_{10} pfu/ml) Lung	Brain	Liver	Kidney
2	HK156-CK	6.7/−[b]	−/−	−/−	−/−
	B1-1-1	6.0/6.6	−/−	−/−	−/−
	B1-1-2	6.9/7.2	−/−	−/−	−/−
	B3-1-1	5.9/6.5	2.1/1.5	−/−	−/−
	HK156-E3	−/−	−/−	−/−	−/−
	L7-4-1	0.7/4.7	−/−	−/−	−/−
	D5-7-1	5.0/5.1	−/−	−/−	−/−
4	HK156-CK	5.8/6.2	−/−	−/−	−/−
	B1-1-1	6.3/6.5	1.3/1.7	−/−	−/−
	B1-1-2	6.0/6.8	1.4/1.6	−/−	−/−
	B3-1-1	6.6/6.8	2.7/2.7	−/−	−/−
	HK156-E3	5.3/5.4	−/−	2.5/−	1.0/−
	L7-4-1	5.4/5.4	−/−	−/−	−/−
	D5-7-1	4.4/4.9	−/−	−/−	−/−
6	HK156-CK	4.5/4.9	3.3/3.1	−/−	−/1.2
	B1-1-1	ND[c]	ND	ND	ND
	B1-1-2	ND	ND	ND	ND
	B3-1-1	ND	ND	ND	ND
	HK156-E3	3.5/4.0	−/−	−/−	−/−
	L7-4-1	4.8/5.0	−/−	−/−	−/−
	D5-7-1	4.2/4.3	−/−	−/−	−/−

[a] Mice were infected with i.n. 2×10^5 pfu of each virus. To determine virus titers, two mice were sacrificed on days 2, 4, and 6 p.i. Virus titers were determined by plaque assay in MDCK cells in the presence of trypsin.

[b] −, virus titer less than 10 pfu/ml.

[c] ND, not determined.

day 6 p.i. from the brain of mice infected i.n. with HK156-CK (Table 3) were cloned in MDCK cells (B1-1-1, B1-1-2, and B3-1-1). Similarly, low pathogenic egg-derived virus HK156-E3 was cloned in chick embryo (CE) cells (L7-4-1 and D5-7-1). Biological differences of parental viruses HK156-CK and HK156-E3 and their respective clones were then compared by determining infectivities in MDCK cells and embryonated eggs (Table 3).

Despite different passage histories of the viruses, all viruses showed similar infectivities in MDCK cells based on virus titers. However, it was noticeable that the sizes of the plaques formed by the viruses tended to vary. For instance, parental virus HK156-CK virus and virus clones of mouse brain isolates, B1-1-1, B1-1-2, and B3-1-1, produced pinpoint and/or medium-sized plaques, whereas HK156-E3 clones (L7-4-1 and D5-7-1) produced only medium-size plaques. Furthermore, all viruses demonstrated comparable infectivities in embryonated hens' eggs (EID_{50}) ranging from titers of $10^{7.5}$ to $10^{8.2}$ pfu/ml. It was thus apparent that even after repeated passaging and cloning of HK156 virus in distinct host systems, there were no appreciable differences in infectivity of these viruses in embryonated eggs or MDCK cells.

Virulence of cloned viruses in eggs and mice

As shown in Table 3, although all viruses grew well in embryonated eggs and showed comparable infectivities (EID_{50}), their virulence in eggs varied considerably. It was apparent that ELD_{50} titers of brain-derived clones (4.0×10^2, 1.0×10^4, and 6.0×10^3) were much higher than those of HK156-CK (0.6) and egg-derived viruses (L7-4-1 and D5-7-1) (0.2). Similarly, virulence of brain-derived virus clones in mice was found to contrast markedly with that of egg-derived virus clones. Clones B1-1-1, B1-1-2, and B3-1-1, which demonstrated low pathogenicity in eggs, showed MLD_{50} values of 3.2 pfu, 250-fold higher than that of HK156-CK (Table 3). Moreover, it was shown that virulence of HK156-E3 cloned viruses in mice ($>2 \times 10^5$ pfu) was appreciably lower than that of parental HK156-CK virus. Furthermore, brain-derived clones were clearly more pathogenic in mice than other HK156-CK clones after i.n. or i.c. infection (Fig. 2). For example, although all mice infected i.n. or i.c. with brain-derived viruses died by day 7 or 8 p.i., all mice infected i.n. with HK156-E3 clones survived, whereas those infected i.c. with 1×10^4 pfu showed 60–80% survival (Fig. 2).

Differences in tissue tropism of cloned viruses

Virus isolation from internal organs of mice infected i.n. (Table 1) revealed that all virus clones examined could replicate in the lungs early as 2 days p.i. Although egg-derived clones appeared to grow more efficiently at day 2 p.i. than their parental strain (HK156-E3), all egg-derived strains grew to similar titers by day 4 p.i. Also, brain-derived clones generally appeared to grow to higher titers than clones derived from eggs. In particular,

TABLE 2

Infectivity of HK156 Viruses Used in the Study in Mouse Brain after i.c. Inoculation[a]

Virus	Virus titer (log_{10} pfu/ml) in mouse brain on days p.i. 2	4	6
HK156-CK	ND[b]	4.2/4.3	ND
B1-1-1	4.3/4.3	4.7/5.0	ND
B1-1-2	3.5/4.7	5.2/5.3	ND
B3-1-1	4.2/4.7	4.9/5.0	ND
HK156-E3	ND	2.5/2.6	ND
L7-4-1	2.7/3.2	3.5/4.5	1.2/2.7
D5-7-1	3.2/3.2	3.5/3.7	−[c]/3.1
Aichi68[d]	ND	−/−	ND

[a] Two mice were infected i.c. with 1×10^4 pfu of each virus. After 2, 4, and 6 days p.i., mouse brain were collected and virus titers were determined by plaque assay in MDCK cells in the presence of trypsin.

[b] ND, not determined.

[c] −, virus titer less than 10 pfu/ml.

[d] Aichi68 was used as a negative control as a non-neurovirulent virus.

TABLE 3

Comparison of Growth Characteristics and Virulence of HK156 Virus and Its Cloned Viruses Used in the Study

Virus	Passage history[a]	Plaque size in MDCK cells[b]	$\log_{10}$ pfu/ml in MDCK cells	EID_{50} ($\log_{10}$/ml)	ELD_{50} (pfu)	MLD_{50} (pfu)
Pathogenic in mice						
HK156-CK	MX/M2	Pinpoint (30%) Medium (70%)	7.3	7.5	0.6	8.0×10^2
Mouse brain clone						
B1-1-1	MX/Ms1M4	Pinpoint (74%) Medium (26%)	7.2	7.5	4.0×10^2	3.2
B1-1-2	MX/Ms1M4	Pinpoint	7.7	7.5	1.0×10^4	3.2
B3-1-1	MX/Ms1M4	Medium	7.8	8.0	6.0×10^3	3.2
Less pathogenic in mice						
HK156-E3	MX/M1E3	Medium	7.3	8.0	0.2	1.3×10^4
HK156-E3 clone						
L7-4-1	MX/M1E3C3E1	Medium	7.6	8.2	0.2	$>2 \times 10^5$
D5-7-1	MX/M1E3C3E1	Medium	7.8	8.0	0.2	$>2 \times 10^5$

[a] Passage history in Hong Kong/our laboratory. MX, MDCK cells (passage number is unknown); M, MDCK cells; Ms, mouse; C, CE cells; E, eggs. The number indicates the number of passages. Method for virus cloning was described in Materials and Methods.

[b] Pinpoint plaque formed <1 mm in diameter; medium plaque formed 1–2 mm in diameter.

marked variability in growth levels of these viruses in brain tissue was observed. In fact, the growth of one clone was detected as early as day 2 p.i., and all high pathogenic clones were isolated from brain tissue by day 4 p.i. In contrast, growth of low pathogenic clones could not be detected in the brain of infected mice.

Because considerable variability among lung virus titers in mice infected with cloned viruses were observed, an additional experiment was undertaken to better characterize the growth profiles of cloned viruses in mouse lung. Ten mice were infected i.n. with 2×10^5 pfu of each clone and examined at 4 days p.i. (Fig. 3). Brain-derived clones, B1-1-1 and B1-1-2, consistently grew to high levels with mean titers ($\log_{10} \pm \log_{10}$SD pfu/ml) of 6.0 ± 0.3 and 6.0 ± 0.2 pfu/ml, respectively, whereas egg-derived clones, L7-4-1 and D5-7-1, grew to appreciably lower mean titers of 3.2 ± 2.1 and 4.1 ± 1.3 pfu/ml, respectively. However, consistent with variable titers observed between two mice infected with egg-derived clone L7-4-1, at 2 days p.i. (Table 1), titers of clones L7-4-1 and D5-7-1 were found to vary considerably, ranging from $<10^1$ pfu/ml to approximately 10^5 pfu/ml (Fig. 3). Nevertheless, observed growth differences between high virulent (B1-1-1 and B1-1-2) and low virulent (L7-4-1 and D5-7-1) cloned viruses in mouse lung were significant ($P < 0.01$).

Similar to parental strains HK156-CK and HK156-E3, all brain- and egg-derived clones were shown to grow in mouse brain after i.c. inoculation (Table 2). These results indicated that although egg-derived clones did not demonstrate neurotropic growth after i.n. infection, these viruses retained their ability to replicate in mouse brain.

Nucleotide and amino acid sequence analysis of HK156 variants

Sequence analysis of the entire genome of HK156-CK was undertaken to understand the genetic basis of observed variable plaque morphology (pinpoint and medium) in MDCK cells. As shown in Table 4, HK156-CK parental virus contained at least two variant populations; two nucleotides were confirmed at two positions in the HA gene and at one position in the PB1 gene, all of which coded for amino acid substitutions. Ser and Asn residues were observed at position 155, whereas Pro and Thr residues were apparent at position 211 of the HA1 domain of the HA protein. Similarly, position 17 of the PB1 protein consisted of an Ala and an Asp residue. Subsequently, nucleotide sequences of the HA gene of a total of 18 cloned viruses were determined to understand whether the above amino acid pairs were conserved at similar positions (data not shown). It was found that 11 of the cloned viruses contained Ser at position 155 of the HA1 domain, whereas the remaining 7 contained Asn at the same position. Also, seven viruses possessed Pro at position 211, although the remaining viruses possessed Thr at the same position. Passaging of HK156-CK in embryonated hens' eggs (HK156-E3) appeared to select for a dominant virus that contained single amino acid residues at positions 155 (Ser) and 211 (Thr) of HA1 domain as well as at position 17 (Ala) of the PB1 protein. Furthermore, additional conserved amino acid substitutions were confirmed in the NS1 ($Asp^{101} \rightarrow Asn$) and PB2 ($Asn^{701} \rightarrow Asp$) proteins.

To investigate which population of HK156-CK virus became dominant in mouse brain after i.n. infection, we sequenced the complete genome of these brain-derived

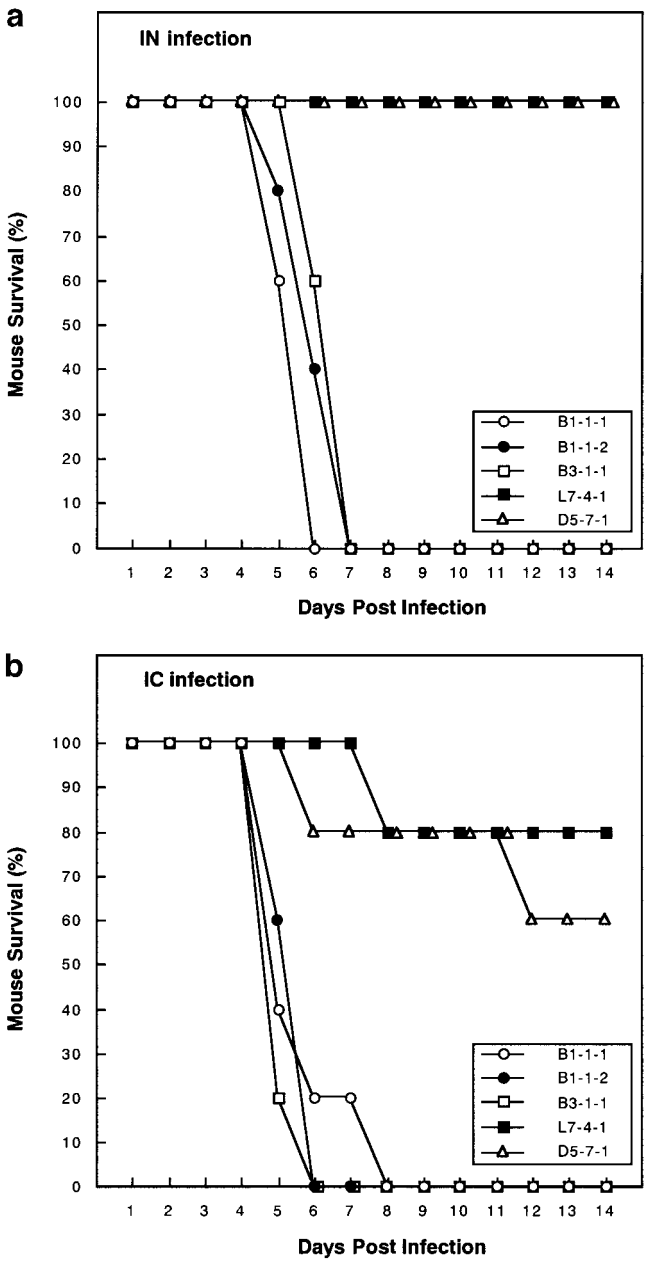

FIG. 2. Survival rates of ddY mice infected with HK156-CK mousse-brain clones and HK156-E3 cloned viruses. Groups of five mice were infected i.n. with 10^5 pfu (a) and i.c. with 1×10^4 pfu (b).

clones. Additionally, viral RNA was extracted from brain homogenate of a mouse infected i.n. with HK156-CK at 6 days p.i., and the complete HA and NS and partial PB1, PB2, PA, and NP genes were sequenced. It became apparent that growth in mouse brain selected for Ser at position 155 and for Pro at position 211 of the HA protein as well as for Ala at position 17 of the PB1 protein. Conserved amino acid substitutions were also revealed in the PB1 ($His^{456} \rightarrow Tyr$ and $Ser^{712} \rightarrow Pro$), PA ($Gly^{631} \rightarrow Ser$), NP ($Glu^{127} \rightarrow Lys$), and PB2 ($Asn^{701} \rightarrow Asp$) proteins of all brain-derived clones and sequences determined directly from mouse brain tissue. It was noteworthy that amino acid variability between brain-derived clones and HK156-E3 cloned viruses was confirmed. All brain-derived clones contained differences in the HA1 (Pro^{211} versus Thr^{211}), NS (Asp^{101} versus Asn^{101}), PB1 (Tyr^{456} versus His^{456}/Pro^{712} versus Ser^{712}), PA (Ser^{631} versus Gly^{631}), and NP (Lys^{127} versus Glu^{127}) proteins. Additional sporadic amino acid changes were also observed in the NA protein of HK156-E3 cloned viruses L7-4-1 (Ile^{54}) and D5-7-1 (Arg^{83}).

In agreement with previous characterization of a series of basic amino acids adjacent to the cleavage site of the connecting peptide in the HA molecule of HK156 virus (Claas *et al.,* 1998; Suarez *et al.,* 1998; Subbarao *et al.,* 1998), all viruses examined in the present study possessed these same basic amino acids. Thus results suggested that changes in virulence of this virus in mice are not dependent on the cleavability of HA protein but possibly are due to other genetic changes.

DISCUSSION

Human H5N1 viruses are reported to have been a direct transmission of an avian virus (Claas *et al.,* 1998; Suarez *et al.,* 1998). HK156 strains have also been shown to cause lethal infection in mice (Gao *et al.,* 1999; Gubareva *et al.,* 1998; Lu *et al.,* 1999; Shortridge *et al.,* 1998). This evidence led us to study the pathogenicity of HK156 in a mouse model. As stated, we initially observed that the pathogenicity of this virus was attenuated in mice after a few passages in embryonated hens' eggs. HK156 is known to replicate in multiple organs (Gao *et al.,* 1999; Gubareva *et al.,* 1998; Lu *et al.,* 1999; Shortridge *et al.,* 1998) and to be highly virulent in mice. Lu *et al.* (1999) reported that HK156 virus passaged only in eggs

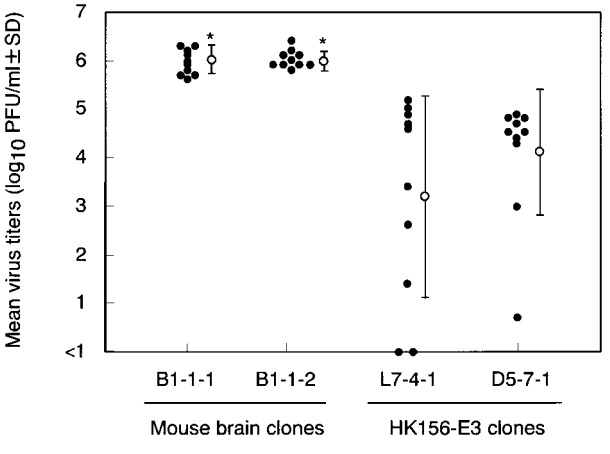

FIG. 3. Replication of HK156-CK mouse brain clones and HK156-E3 cloned viruses in mouse lung after i.n. infection on day 4 p.i. Mice were infected i.n. with 2×10^5 pfu of each virus. To determine virus titers in lungs, 10 mice were sacrificed, and virus titers were determined by plaque assay on MDCK cells in the presence of trypsin. *Differences of virus titers between mice infected with brain-derived clones and those infected with HK156-E3 clones (L7-4-1 or D5-5-1) were significant ($P < 0.01$).

TABLE 4

Comparison of Amino Acid Sequences of HK156 Viruses Used in the Study[a]

	Amino acid at position (nucleotide at position)										
	HA		NS1	PB1			PA	NP	PB2	NA	
Virus	155 (464)	211 (631)	101 (301)	17 (50)	456 (1366)	712 (2134)	631 (1891)	127 (379)	701 (2101)	54 (161)	83 (248)
Pathogenic for mice											
HK156-CK	Ser (G)[b] or Asn (A)	Pro (C)[b] or Thr (A)	Asp (G)	Ala (C)[b] or Asp (A)	His (C)	Ser (T)	Gly (G)	Glu (G)	Asn (A)	Asn (A)	Lys (A)
B1-1-1	Ser (G)	Pro (C)	*	Ala (C)	Tyr (T)	Pro (C)	Ser (A)	Lys (A)	Asp (G)	*	*
B1-1-2	Ser (G)	Pro (C)	*	Ala (C)	Tyr (T)	Pro (C)	Ser (A)	Lys (A)	Asp (G)	*	*
B3-1-1	Ser (G)	Pro (C)	*	Ala (C)	Tyr (T)	Pro (C)	Ser (A)	Lys (A)	Asp (G)	*	*
Mice brain homogenate[c]	Ser (G)	Pro (C)	*	Ala (C)[d]	Tyr (T)[d]	Pro (C)[d]	Ser (A)[d]	Lys (A)[d]	Asp (G)[d]	—[e]	—
Less pathogenic for mice											
HK156-E3	Ser (G)	Thr (A)	Asn (A)	Ala (C)	*	*	*	*	Asp (G)	*	*
L7-4-1	Ser (G)	Thr (A)	Asn (A)	Ala (C)	*	*	*	*	Asp (G)	Ile (T)	*
D5-7-1	Ser (G)	Thr (A)	Asn (A)	Ala (C)	*	*	*	*	Asp (G)	*	Arg (G)

[a] No amino acid substitutions were found in M1, M2, or NS2 proteins. *Identical amino acids.
[b] Mixture of nucleotides was observed in sequence gel image.
[c] Sequences were determined directly from mouse brain homogenate infected i.n. with HK156-CK on 6 days p.i.
[d] Partial sequence was determined in this region.
[e] —, not determined.

was found to replicate in mouse brain and to cause lethal infection in mice after i.n. infection. In the present study, however, HK156-CK virus passaged in eggs became less virulent in mice and could not be detected in brain tissue after i.n. infection. A possible reason for these contrasting results may be the independent selection of low and high virulent viruses from a mixed population through passaging in eggs. A direct comparison of HK156 variants passaged in eggs by Lu *et al.* (1999) and in our laboratory through an analysis of acquired mutations in the genes of these viruses may help elucidate the mechanisms for apparent pathogenic variability.

It has been reported that plaque size of Hong Kong H5N1 viruses in MDCK cells correlated with observed pathogenic variability among these viruses in mice (Gao *et al.,* 1999). This correlation was also apparent in this study as high pathogenic viruses in mice produced pinpoint and/or medium-size plaques, whereas less pathogenic viruses produced only medium-size plaques. A previous study suggested that amino acid residue 627 in the PB2 protein of Hong Kong H5N1 viruses may be associated with plaque formation in MDCK cells (Gao *et al.,* 1999). However, plaque heterogeneity among viruses examined in this study did not appear to be associated with changes at this position. Amino acid changes in the HA1 (residue 211), PB1 (residues 456 and 712), PA (residue 631), NP (residue 127), and NS1 (residue 101) proteins were identified that may affect plaque formation in MDCK cells. Nevertheless, it was unexpected to observe that viruses B1-1-1, B1-1-2, and B3-1-1, which demonstrated distinct plaque phenotypes and variable pathogenicity in eggs, contained identical protein amino acid sequences. Because sequence analysis of these viruses was made after plaque assays and egg pathogenicity experiments, it is not possible that variable viruses arose after sequencing of their genomes. Mutation analyses of viral RNA have shown that changes in the conserved noncoding double-stranded region may affect virus infectivity (Fodor *et al.,* 1998). Although noncoding terminal sequences were not compared in this study, the possibility of mutations in this region may account for observed growth differences among HK156 viruses.

HK156-CK (MDCK line) and its brain-derived clones were detected in mouse brain after i.n. infection, whereas egg-derived viruses were not. However, it was noteworthy to reveal that this egg-derived virus and its cloned viruses were able to replicate in brain after direct i.c. infection. In contrast, HK156-CK and its brain-derived cloned viruses grew well in brain after either i.n. or i.c. infection. This evidence indicated that HK156-CK and its brain-derived clones demonstrated a neurotropic phenotype that evidently allowed these viruses to migrate from the lungs to the brain. On the other hand, despite demonstrating limited replication and neurovirulence in mice after i.c. infection, egg-passaged viruses appeared to lose their capacity to disseminate from the lungs to the brain.

How are these differences in virulence related to changes in the structural and nonstructural proteins of these viruses? Conserved amino acid differences in the HA1 domain of the HA protein and internal PB1, PA, NP, and NS1 proteins were identified between high and low pathogenic viruses. Low pathogenic viruses showed amino acid changes in the HA1 domain (Pro/Thr^{211}→Thr) and in the NS1 protein (Asp^{101}→Asn). Indeed, HK156 viruses that possessed Thr^{211} in the HA1 domain and

Asn[101] in the NS1 protein appeared to be less virulent in mice after i.n. or i.c. infection. It has been reported that biological heterogeneity among Hong Kong H5N1 viruses isolated from humans was observed based on pathogenicity in a mouse model (Gao *et al.,* 1999; Lu *et al.,* 1999). Because these viruses all possessed multiple basic amino acids at the cleavage site of the HA protein (Claas *et al.,* 1998; Suarez *et al.,* 1998; Subbarao *et al.,* 1998), it was suggested that viral pathogenicity in mice appeared to be dependent on factors other than HA cleavability. Consistent with these reports, high and low pathogenic viruses analyzed in the present study all possessed multiple basic amino acids at the HA cleavage site, suggesting that cleavability of the HA protein is not an essential determinant of pathogenicity in mice. Results of sequence analysis in this study suggested that mutations at position 211 of the HA1 domain and/or 101 of the NS1 protein of HK156 virus correlated with attenuation of this virus in mice. Also, mutations in the PB1, PA, and NP proteins, which were also identified in viral RNA extracted from mouse brain, may be associated with neurovirulence in mice.

Even though host cell-mediated variation has been well recognized as a selection mechanism for HA mutants (Gubareva *et al.,* 1998; Ito *et al.,* 1997b; Katz *et al.,* 1987; Meyer *et al.,* 1993), these mutations are known to occur in the receptor-binding domain of the HA molecule. The Thr at position 211 of the HA protein is a unique feature of HK156-E3 among avian H5 influenza viruses, including strains isolated from chickens (5 viruses) and humans (16 viruses) from 1997 to 1998 in Hong Kong (Bender *et al.,* 1999; Claas *et al.,* 1998; Suarez *et al.,* 1998; Subbarao *et al.,* 1998). Also, in addition to all H5 HA protein sequences available from GenBank, 11 other HA serotypes (H1, H2, H3, H4, H6, H7, H8, H9, H10, H11, and H12) (Nobusawa *et al.,* 1991) were also found to possess Pro at this position. Based on the characterized structure of the H3 HA molecule (Wilson *et al.,* 1981), it was evident that amino acid residue 211 is located near the receptor-binding region of the HA molecule. Philpott *et al.* (1990) suggested that attenuation and altered cell tropism of highly pathogenic H5 avian virus in chickens were caused by an escape mutation in the HA molecule (Philpott *et al.,* 1990). Although there were no considerable differences of receptor specificity observed based on hemagglutination patterns among HK156 viruses with erythrocytes from different animal species (data not shown), a mutation at position 211 of the HA1 domain may alter the functionality of HA molecule or cell tropism.

Nevertheless, parental HK156-CK appeared to consist of a mixed population of HA1 and PB1 proteins. Viruses containing Ser[155] and Pro[211] in the HA1 domain became dominant among viruses isolated from brain after i.n. infection of HK156-CK. Additional amino acid changes among brain isolates were also determined in the PB1(His[456]→Tyr, Ser[712]→Pro), PA (Gly[631]→Ser), NP (Glu[127]→Lys), and PB2 (Asn[701]→Asp) proteins. Mouse brain-cloned viruses that possessed these amino acid substitutions became more virulent in mice than the parental HK156-CK strain. A comparison of amino acid sequences of high and low pathogenic viruses indicated that specific amino acid changes in the HA1 domain of the HA protein (residue 211), as well as the PB1 (residues 456 and 712), PA (residue 631), and NP (residue 127) proteins, appeared to correlate with adaptation and virulence in mice. In addition, amino acid sequences of brain homogenate of mouse infected i.n. with HK156-CK suggested that amino acid changes in the PB1 (residues 456 and 712), PA (residue 631), and NP (residue 127) proteins may be associated with neurovirulence in mice. Moreover, high pathogenicity of HK156-E3 and its clones in eggs suggested that these amino acid residues may also be involved in virulence in chickens. Furthermore, observed pathogenic variability between HK156-E3 and clones L7-4-1 and D5-7-1 appeared to be related to sporatic amino acid changes in the NA protein of L7-4-1 (Ile[54]) and D5-7-1 (Arg[83]).

In this study, we revealed that passaging of HK156 in mouse brain and embryonated eggs led to the selection of high and low virulent variants in the mice model, respectively. These variant phenotypes correlated with specific amino acid changes in the HA, PB1, PA, NP, and NS1 proteins, indicating that virulent phenotypes of H5N1 viruses were modified by the use of different culture systems. Further study on mutations in the HA protein as well as other proteins may provide evidence that explains the mechanisms of virulence or neurovirulence of avian influenza virus in mice and other mammals.

MATERIALS AND METHODS

Viruses and cells

MDCK cell-derived HK156 was kindly provided by Dr. W. Lim (Virus Unit, Queen Mary Hospital, Hong Kong). Seed viruses were prepared in either MDCK cells (37°C, 48 h) or allantoic cavities of 10-day-old embryonated hens' eggs (35°C, 48 h). Early human H3N2 A/Aichi/2/68 (Aichi68) was propagated in allantoic cavities of 10-day-old embryonated hens' eggs (34°C, 48 h). The 50% egg infectious dose (EID_{50}) and egg lethal dose (ELD_{50}) infectivities were determined by inoculating serial 10-fold dilutions of virus into eggs followed by observation after a 4-day incubation. Cloned viruses B1-1-1, B1-1-2, and B3-1-1 were prepared from mouse brain homogenates after i.n. infection with HK156-CK, which were then plaque purified three times in MDCK cells. These purified viruses were finally propagated once in MDCK cells. HK156-E3 clones L7-4-1 and D5-7-1 were prepared through three sequential plaque purification in CE cells, and seed viruses were finally propagated once in allantoic cavities of 10-day-old embryonated hens' eggs. The viruses used in this study and their passage histories are

listed in Table 3. MDCK cells were cultured in minimal essential medium (MEM) containing 10% fetal calf serum (FCS). Primary CE cells were prepared from 5-day-old embryonated hens' eggs and maintained in MEM with 5% FCS.

All experiments with live HK156 virus were carried out in a BL3 laboratory at the National Institute of Infectious Diseases (Tokyo, Japan), and research personnel were administered amantadine for prevention against infection as prescribed by a physician.

Animal experiments

Five-week-old specific pathogen-free (SPF) female ddY mice (Japan SLC Inc., Shizuoka, Japan) were used in all experimental infections. Before infection, mice were anesthetized by intraperitoneal administration of pentobarbital sodium (Nembutal) and inoculated i.n. or i.c. with 10 or 50 μl of virus suspension, respectively, diluted in PBS containing 1% bovine albumin. The 50% mouse lethal dose (MLD_{50}) titers and survival experiments were determined by i.n. inoculation of groups of five mice with serial 10-fold virus dilutions that were observed until 13 or 14 days postinfection (p.i.).

To determine virus infectivity in organs, mice were infected i.n. or i.c. with 2×10^5 and 1×10^4 pfu of viruses, respectively. Mice were sacrificed at 2, 4, and 6 days p.i., and organs were removed in the order of brain, kidney, liver, and lung. Each organ was homogenized to make 10% homogenates (w/v) in PBS and frozen at $\leq$83°C until plaque titration. Virus titers in organs were determined in MDCK cells in the presence of trypsin and incubated for 2 days at 37°C, followed by calculation of pfu values on the next day.

Sequence analysis

Viral RNA was extracted from culture fluid of MDCK cells, allantoic fluids, or mouse brain homogenates with RNeasy extraction kit (Qiagen Corp., Chatsworth, CA). RT–PCR was performed using a modified protocol of a commercial kit (RT–PCR kit with avian myoblastosis virus reverse transcriptase, version 2.1; Takara, Kyoto, Japan) as described previously (Lindstrom *et al.,* 1998). RT reactions were performed using a previously described universal influenza A primer (5′-AGCAAAAGCAGG-3′) (Zou, 1997). Resultant cDNAs were used in all subsequent PCR amplifications of overlapping cassettes covering the entire protein coding domain of all genes by using the following thermocycler program: initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen Corp.) and sequenced directly with synthetic oligonucleotide primers using Thermo Sequenase II dye terminator cycle sequencing premix kit (Amersham Pharmacia Biotech Inc.) on a model 377 or 310 (Perkin–Elmer Cetus, Applied Biosystems Inc., Norwalk, CT) DNA autosequencer.

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